EXTENDED REPORT

Age-related differences in the normal human cornea: a laser scanning in vivo confocal microscopy study

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Br J Ophthalmol 2007;**91**:1165–1169. doi: 10.1136/bjo.2006.112656

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Accepted 1 March 2007
Published Online First 27 March 2007

Aims: To quantify and establish baseline normative data for age-related differences in cellular and innervation density in the normal, healthy, human cornea using laser scanning in vivo confocal microscopy.
Methods: Cross-sectional study of 85 normal subjects assessed via corneal topography and laser scanning in vivo confocal microscopy.
Results: Mean age was 38±16 years (range 18–87 years) and 60% of subjects were female. Anterior keratocyte density declined by 0.9% per year ($r=-0.423$, $p<0.001$), posterior keratocyte density declined by 0.3% per year ($r=-0.250$, $p=0.021$) and endothelial cell density declined by 0.5% per year ($r=-0.615$, $p<0.001$). Sub-basal nerve fibre density declined by 0.9% per year ($r=-0.423$, $p<0.001$). No association was observed between age and basal epithelial cell density, or between age and central corneal thickness, corneal astigmatism or horizontal corneal diameter ($p>0.05$). No association was observed between subject gender and corneal cell or innervation density.
Conclusions: Using laser scanning in vivo confocal microscopy this study highlights a significant, and relatively linear, reduction in keratocyte and endothelial cell density with increasing subject age. Interestingly, corneal sub-basal nerve fibre density also significantly decreases with increasing age. In vivo laser scanning confocal microscopy provides a safe, non-invasive method for the establishment of normative data and assessment of alterations in human corneal microstructure following surgery or disease processes.

Aging generates structural and functional changes in the cornea and has been associated with corneal steepening, a shift from with-the-rule to against-the-rule astigmatism and increased thickness of Descemet's membrane.[1] Corneal wound healing has been observed to decline with age and age affects refractive outcomes and final visual acuity following laser refractive surgery.[2–5] Specular microscopy has demonstrated a gradual decrease in endothelial cell density with age, accompanied by an increase in endothelial cell size, increased coefficient of variation and a decreased proportion of hexagonal cells,[6] but, until relatively recently, visualisation of other cell layers in the living human cornea has remained elusive. Over the past decade, the introduction of in vivo confocal microscopy has provided a new method for corneal examination at high resolution in living patients through optical sectioning. This technique has enabled microstructural analysis of the normal human cornea, under more physiological conditions than previously possible.[7]

The aim of this study was to use a laser scanning in vivo confocal microscope to quantitatively analyse the basal epithelium, stroma, endothelium and sub-basal nerve plexus in the normal human cornea across a broad age range.

MATERIALS AND METHODS

Subject recruitment and assessment

A total of 85 normal subjects were prospectively recruited from the Department of Ophthalmology, Auckland City Hospital and analysed in the Department of Ophthalmology, University of Auckland. Subjects were invited to participate if they were aged 16 years or over, with the following as criteria for exclusion: history of ocular surgery, current or long-term prior topical ocular medication, previous or active ocular pathology (other than refractive error) and prior contact lens wear. Study protocol required parental consent for subjects aged under 18 years. Prescription systemic medications were not exclusion criteria unless they were known to affect the cornea or anterior segment. All subjects were examined by high-magnification slit lamp biomicroscopy and their corneas were confirmed to be clinically normal prior to inclusion in the study. Corneal topography and central corneal thickness were measured using the Orbscan II slit-scanning elevation topography system (Bausch & Lomb Surgical, Rochester, New York, USA).

This study received approval from the Auckland Ethics Committee and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects following detailed explanation of the nature of the study.

Measurements were performed using a calliper tool (analySIS V.3.1, Soft Imaging System, Münster, Germany). For all basal epithelial and endothelial pictures a standard central counting frame size of 200 μm ×200 μm was used. This counting frame was applied to the centre of the confocal image and the position was constant for every image analysed. For all sub-basal nerve plexus and stromal images the full 400 μm ×400 μm frame was used. Keratocyte cell density was assessed by counting all bright images with clear borders within the counting frame. Nerve fibre density (NFD) was assessed by measuring the total length of all nerve fibres and branches per square millimetre.

In vivo confocal microscopy

Laser scanning in vivo confocal microscopy was performed on all subjects with the HRT II corneal module that uses a 670 nm red wavelength diode laser source (Heidelberg Retina Tomograph II Rostock Corneal Module (RCM); Heidelberg Engineering GmbH, Heidelberg, Germany). A 60× objective water immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan) and a working distance, relative to the applanating cap, of 0.0–3.0 mm was used. The images produced using this lens are 400 μm ×400 μm and the manufacturer quotes the transverse resolution and optical

Abbreviations: LASIK, laser-assisted in-situ keratomileusis; NFD, nerve fibre density; PRK, photorefractive keratectomy; RCM, Rostock corneal module

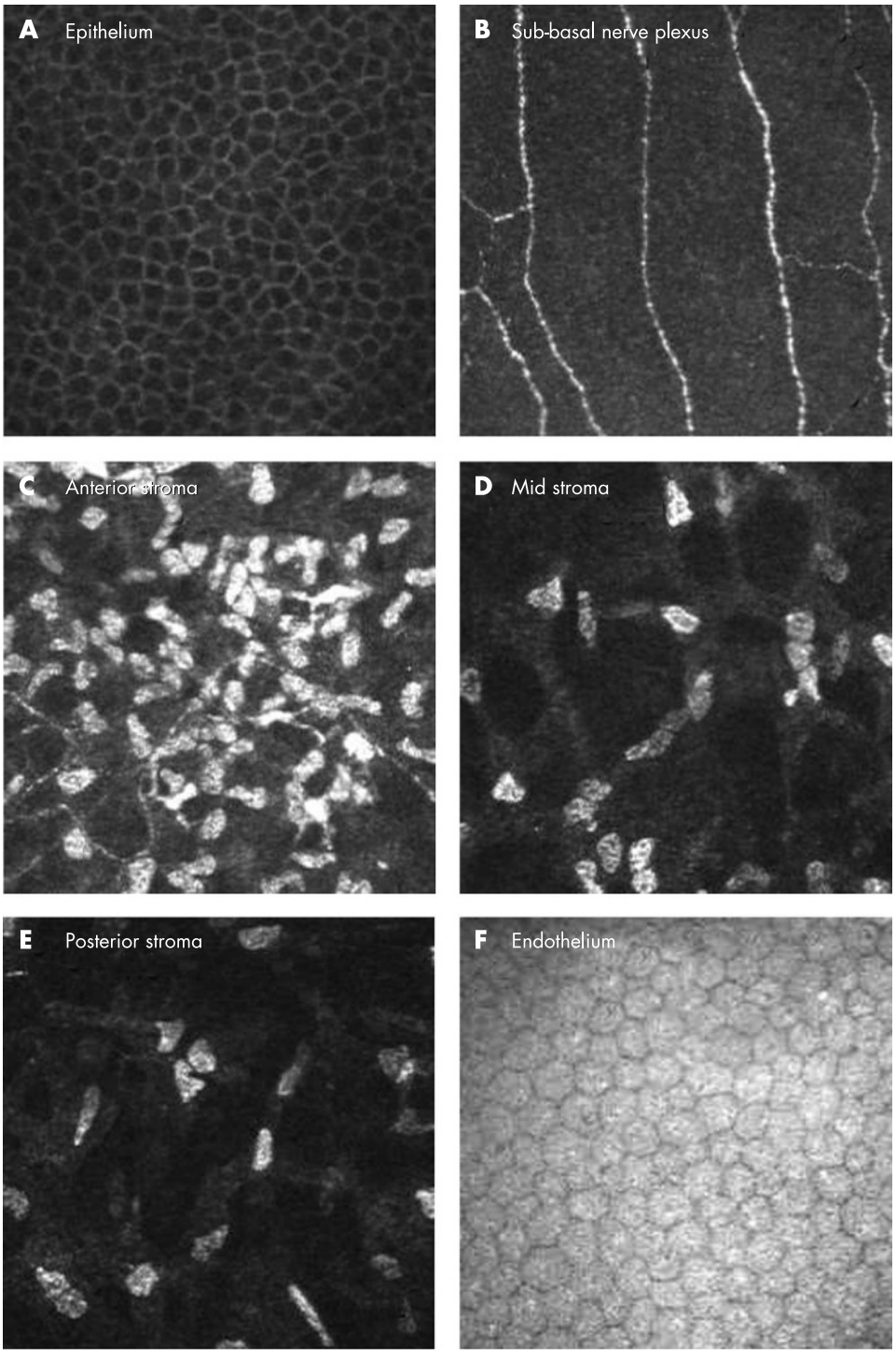

Figure 1 Laser scanning in vivo confocal microscopy of the normal human cornea demonstrating characteristic features. Images cropped to 250 μm ×250 μm.

section thickness as 2 μm and 4 μm, respectively. The RCM uses an entirely digital capture system.

The central cornea of the right eye was examined in each subject. Each eye was anaesthetised with one drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Surrey, UK). Viscotears (Carbomer 980, 0.2%; Novartis, North Ryde, New South Wales, Australia) was used as a coupling agent between the applanating lens and the cornea. All subjects were asked to fixate on a distance target aligned to enable examination of the central cornea. The full thickness of the central cornea was scanned using the device's "section" mode. The total duration of in vivo confocal examination was approximately 2 min/eye. None of the subjects experienced any visual symptoms or corneal complications as a result of the examination.

Image analysis

For each cornea, three images were taken from each of the following levels; basal epithelium, sub-basal nerve plexus, anterior stroma, mid stroma, posterior stroma and endothelium (fig 1). Anterior stroma was defined as the first three clear images (without motion blur or compression lines) immediately posterior to Bowman's layer, posterior stroma was defined as the first three clear images immediately anterior to Descemet's membrane and mid stroma was defined as three images equidistant from Bowman's layer and Descemet's membrane in the full thickness section. The z distance between images was 2 μm. All images were subsequently randomised and encoded by a single independent observer (DP).

Table 1 Subject characteristics (n = 85)

Characteristic	Value
Age (years)	37.8 ± 16.4
Sex:	
Male	34 (40%)
Female	51 (60%)
Ethnicity:	
Caucasian	62 (73%)
Asian	17 (20%)
Other	6 (7%)
Central corneal thickness (μm)	555 ± 31.0
Horizontal corneal diameter (mm)	11.8 ± 0.40

Age, central corneal thickness and horizontal corneal diameter are expressed as mean ± standard deviation

Statistical analysis

All values were entered into a Microsoft Excel database and subsequently imported into statistical software for analysis. Statistical analysis was performed in SPSS V.12 for Windows (Chicago, Illinois, USA). Basic descriptive statistics were calculated on all data gathered and values are reported as mean ± standard deviation. Normal distribution of cell and innervation density was determined using the Kolmogorov–Smirnov test.[8] Correlations between continuous variables were examined by calculating Pearson's correlation coefficient (r). Student's independent t-test was used to compare values between two groups and comparison between cell layers was calculated with a paired t-test. All tests were two-tailed and a P value of less than 0.05 was considered statistically significant.

RESULTS

Results from in vivo confocal microscopy of 85 normal corneas were included for analysis. The mean age of subjects was 37.8 years ± 16.4 (range 18–87 years). Mean CCT was 555 ± 31.0 μm. Subject characteristics are reported in table 1. Mean cell densities were; basal epithelium 6000 ± 1080 cells/mm^2, anterior stroma 765 ± 262 cells/mm^2, mid stroma 347 ± 64.4 cells/mm^2, posterior stroma 315 ± 57.2 cells/mm^2 and endothelium 2720 ± 367 cells/mm^2. Mean sub-basal nerve fibre density (NFD) was 20.3 ± 6.50 mm/mm^2.

No association was observed between subject gender and cell density at the level of the basal epithelium (p = 0.549), anterior stroma (p = 0.583), mid stroma (p = 0.398), posterior stroma (p = 0.288) or endothelium (p = 0.691). No association was observed between subject gender and sub-basal nerve fibre density (p = 0.722), CCT (p = 0.448), corneal astigmatism (p = 0.973) or horizontal corneal diameter (p = 0.544).

Anterior stromal, mid stromal and posterior stromal keratocyte density and endothelial cell density declined with subject age (table 2). Sub-basal nerve fibre density also declined with age, however, no association was observed between basal epithelial cell density and age (table 2). No association was observed between subject age and CCT (r = 0.148, p = 0.176), corneal astigmatism (r = 0.149, p = 0.172) or horizontal corneal diameter (r = −0.020, p = 0.857).

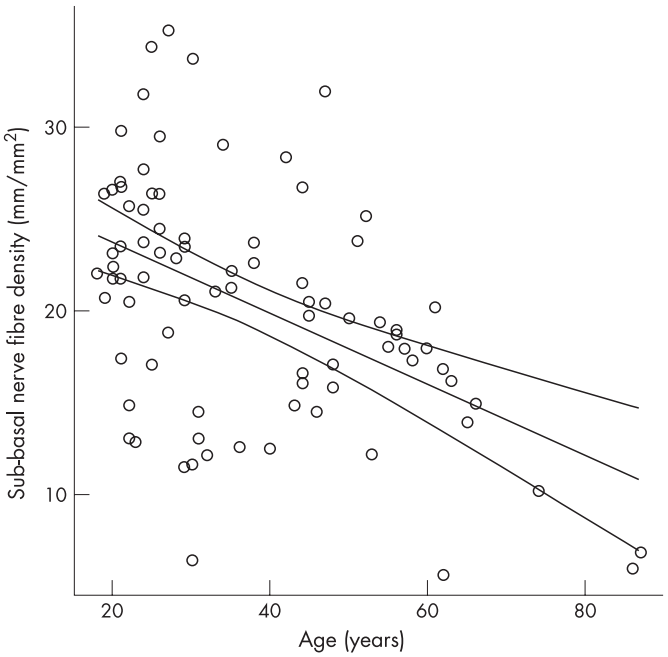

Figure 2 Sub-basal nerve fibre density plotted against increasing age (n = 85 subjects, age range 18–87 years). Sub-basal nerve fibre density measured with in vivo confocal microscopy declines with age of subject (r = −0.488, p<0.001 Pearson's correlation). Linear regression line with 95% mean prediction interval.

Sub-basal NFD reduced by approximately 0.9% per year (fig 2). The three oldest subjects (aged >70 years) had low sub-basal nerve densities. However, when these cases were excluded a significant correlation was still observed between age and sub-basal nerve plexus density (r = −0.367, p = 0.001) but the regression line was slightly less steep, with nerve fibre density reducing by 0.7% per year. A significant, but modest, association was observed between NFD and anterior stromal (r = 0.227, p = 0.037), mid stromal (r = 0.271, p = 0.012) and posterior stromal (r = 0.237, p = 0.029) keratocyte density and between NFD and endothelial cell density (r = 0.331, p = 0.002). No association was observed between NFD and epithelial cell density (r = −0.080, p = 0.467).

Keratocyte density declined by 0.9% per year in the anterior stroma, 0.3% per year in the mid stroma and 0.3% per year in the posterior stroma. Keratocyte cell density was highest in the anterior stroma and lowest in the posterior stroma (765 ± 262 cells/mm^2 versus 315 ± 57.2 cells/mm^2, p<0.001) (fig 3). The in vivo confocal microscopy appearance of the keratocytes differed between anterior and posterior stroma (fig 1C and E, respectively) with keratocyte nuclei appearing smaller in the anterior stroma than in the posterior stroma. A fine network of nerves, comprising the subepithelial nerve plexus, was frequently imaged in the

Table 2 Correlations between epithelial, keratocyte, and endothelial cell densities and sub-basal nerve fibre innervation density with increasing age (Pearson's correlation)

	Correlation*	p Value*
Basal epithelial density (cells/mm^2)	0.200	0.067
Sub-basal nerve fibre density (mm/mm2)	−0.488	<0.001
Anterior keratocyte density (cells/mm^2)	−0.423	<0.001
Mid keratocyte density (cells/mm^2)	−0.237	0.029
Posterior keratocyte density (cells/mm^2)	−0.250	0.021
Endothelial density (cells/mm^2)	−0.615	<0.001

(n = 85 corneas)

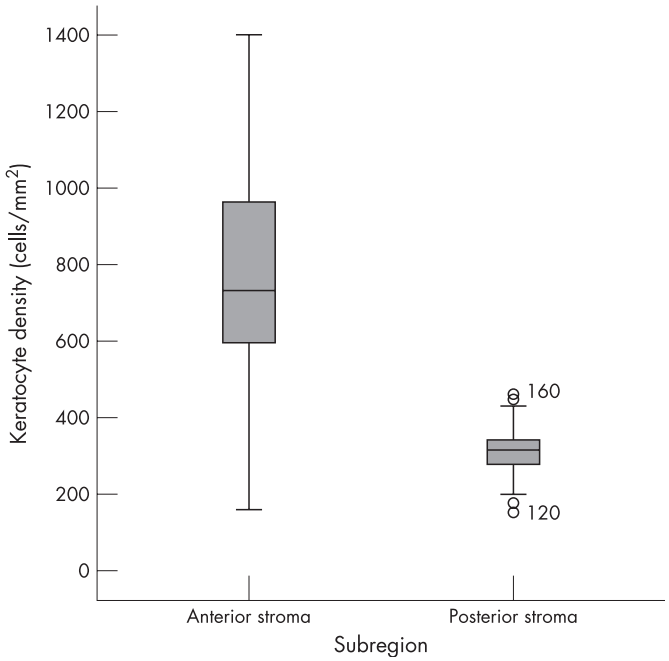

Figure 3 Anterior and posterior keratocyte density. Subregional keratocyte density measured by in vivo confocal microscopy. Anterior keratocyte density was significantly higher than posterior keratocyte density ($p<0.001$).

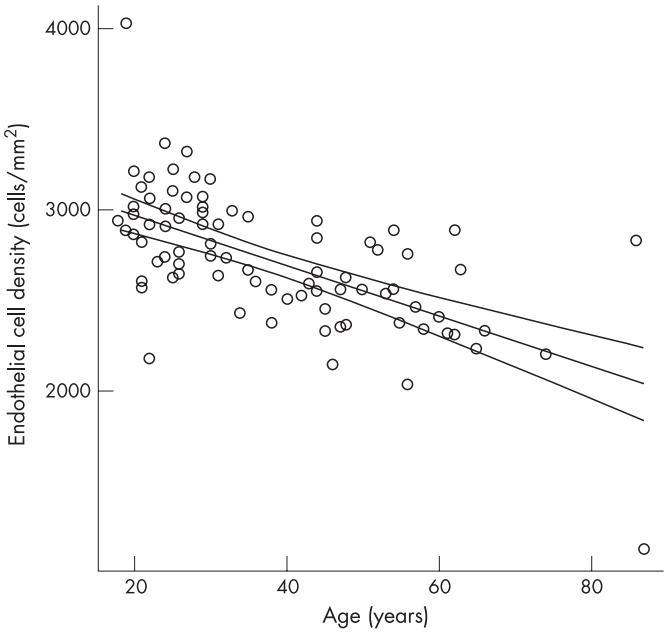

Figure 4 Endothelial cell density plotted against increasing age (n = 85 subjects, age range 18–87 years). Endothelial cell density measured with in vivo confocal microscopy declines with age of subject ($r = -0.615$, $p<0.001$ Pearson's correlation). Linear regression line with 95% mean prediction interval.

anterior stroma. Nuclei were elliptical in the anterior stroma and became more elongated towards the periphery. Significant correlation was observed between anterior stromal and mid stromal keratocyte density ($r = 0.376$, $p<0.001$ paired t-test) and between anterior stromal and posterior stromal keratocyte density ($r = 0.311$, $p = 0.004$ paired t-test).

Endothelial cell density reduced by 0.5% per year (fig 4). Endothelial cell density correlated with CCT ($r = -0.280$, $p = 0.009$ Pearson's correlation). Endothelial guttata were detected by in vivo confocal microscopy in the central cornea of three patients (3.5%) with no associated stromal or epithelial oedema. The subjects were aged 44, 56 and 61 years and all were female. Corneal guttata appeared as a round central mass surrounded by a darker ring at the level of the endothelium (fig 5). No statistically significant difference was observed in keratocyte density in subjects with corneal guttata.

DISCUSSION

The cornea is the most densely innervated of all human tissues, with nerve densities 300–600 times that of skin and 20–40 times that of tooth pulp.[9] Previous studies of changes in corneal innervation density and morphology with age have been limited by poor availability of healthy corneal tissue for ex vivo analysis, and the rapidity of corneal nerve degeneration after death.[10] In vivo confocal microscopy has enabled images of corneal nerve fibre bundles to be obtained by optical sectioning and the en face images are ideally suited to analysis of sub-basal nerve fibre architecture.[7 11] Corneal sensation decreases with age,[12 13] however, there is disagreement between studies as to whether sub-basal nerve fibre density (NFD) declines with age.[11 14] In the current study, utilising a laser scanning in vivo confocal microscope, we observed a statistically significant reduction in NFD with age. Corneal innervation provides important protective and trophic functions for the cornea and a reduction in corneal innervation could result in delayed wound healing.[15 16] Whilst we observed no significant alterations in epithelial cell density with age, the reduction in sub-basal nerve fibre density might explain slower closure of epithelial defects with age.[5]

Keratocyte density has been reported to reduce with age in an ex vivo study and this reduction in keratocyte density with age has subsequently been confirmed by in vivo confocal microscopy studies.[17 18] The current study identified significantly higher keratocyte density in the anterior than posterior stroma and a significant reduction in anterior keratocyte density with increasing age. Studies in humans and rabbits illustrate higher cell density in the anterior stroma and electron microscopy reveals that in the anterior stroma keratocytes contain twice as

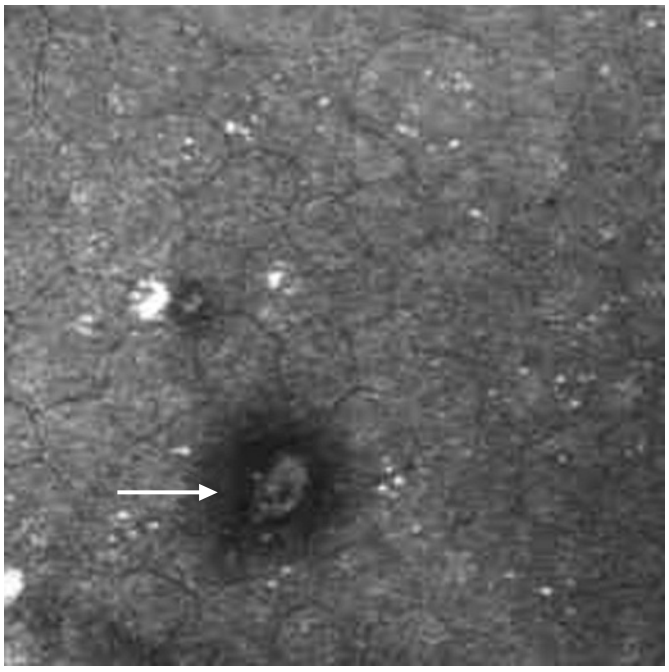

Figure 5 Endothelial guttata. In vivo confocal microscopy of human endothelium. Image cropped to 250 μm ×250 μm. Endothelial guttata marked with white arrow.

many mitochondria as in the mid or posterior stroma.[19] The dense keratocyte cell layer, approximately 100 μm thick, observed immediately posterior to Bowman's membrane, has been identified to contain a morphologically distinct subpopulation of keratocytes with a granular appearance and extensive cytoplasmic cell processes.[20] The significance of the faster reduction in anterior stromal keratocyte density is unclear, but it might represent a reduction in stromal metabolic activity with age, or, alternatively, a change in the composition of keratocyte subpopulations. It is also possible that the apparent reduction in cell density with age might represent true loss of keratocytes or might represent an optical artefact due to decreased image contrast in older subjects. As CCT was not associated with age in this study, the apparent reduction in keratocyte density is unlikely to represent binomial expansion.

Two-dimensional keratocyte density calculated from laser scanning in vivo confocal microscopy (RCM) images of the corneal stroma demonstrate a slightly lower cell density in the anterior and posterior stroma than reported with slit scanning confocal microscopy.[21 22] This difference could be due to the decreased thickness of the optical section taken with the RCM compared to other devices.[23]

Alterations in keratocyte density with age might be associated with reported changes in corneal thickness, keratometry and light scattering properties with age.[1] Recent studies have led to the realisation that keratocytes are highly active cells and could play an important role in better understanding myopic shift, corneal wound healing and post-surgical haze.[24 25] A reduction in keratocyte cell density has been observed in long-term follow-up of subjects following photorefractive keratectomy (PRK)[26] and laser-assisted in-situ keratomileusis (LASIK)[27] and following penetrating keratoplasty.[28] Corneal wound healing response is of particular interest when contemplating surgery in an older age group, as age has been identified as an important factor affecting stromal wound healing following refractive surgery.[4] Further studies investigating the association of keratocyte density in corneal wound healing are warranted.

The reduction in endothelial cell density with age has been well documented in specular microscopy studies[6] and Bourne *et al* quote a linear reduction of 0.6% per year, very similar to the reduction of 0.5% per year observed in this study utilising in vivo confocal microscopy. Central corneal guttata were observed in 3.5% of subjects in this study, occurring in subjects aged 40 years or over. The incidence of corneal guttata in healthy corneas varies between studies and with method of examination. Ex vivo microscopy revealed an incidence of 15% in 182 corneal donors, although this represented an older age group and the incidence of corneal guttata was 0% in the 29 corneas examined from subjects aged less than 50 years.[29] Slit lamp examination of 1,016 subjects observed central corneal guttata in 32% of subjects aged 10–39 and in 70.4% of subjects aged 40–99[30] and, more recently, an in vivo confocal microscopy study observed central corneal guttata in 6–29% of patients aged 60 years or over, but in no patients aged under 60 years.[18] Corneal guttata can be associated with Fuchs' endothelial dystrophy, interstitial keratitis, macular dystrophy, posterior polymorphous dystrophy, trauma, toxins or infection.[31] Identification of central corneal guttata might be important in patients undergoing refractive surgery, as mild corneal guttata has been associated with increased risk of transient corneal oedema, loss of best corrected visual acuity, endothelial cell loss and myopic regression after routine LASIK surgery.[32]

In conclusion, in the current study, in vivo confocal microscopy reveals a reduction in cell density and corneal innervation in the aging cornea. Such alterations might have important implications for corneal structure and function and further studies are necessary to investigate the role of these changes in corneal wound healing and clinical outcomes following corneal surgery.

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Competing interests: None declared

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